pHrodo™ BioParticles™ Phagocytosis Kits for Flow Cytometry

Catalog Numbers A10025, P35381, and P35382

Pub. No. MAN0001950 **Rev.** B.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

pHrodo™ BioParticles™ Phagocytosis Kits for Flow Cytometry offer an outstanding approach for assessing phagocytic activity in whole blood samples by flow cytometry. The pHrodo™ BioParticles™ phagocytosis assays provide sensitive detection without the need for quenching reagents and extra wash steps. The kits include all of the reagents required for assessing particle ingestion and red blood cell lysis. Each kit contains sufficient reagents for performing approximately 100 assays using 100 µL of whole blood per assay.

The pHrodo™ BioParticles™ conjugates are inactivated, unopsonized *E. coli* or *S. aureus* reagents, which are highly sensitive, fluorogenic particles for the detection of phagocytic ingestion. The unique pHrodo™ dye-based system measures phagocytic activity based on acidification of particles as they are ingested, eliminating the wash and quenching steps that are necessary with nonfluorogenic indicators of bacterial uptake (Sahlin, 1983; Wan, 1993). To achieve this, the particles are conjugated to the pHrodo™ dyes, novel fluorogenic reagents that dramatically increase in fluorescence as the pH of their surroundings becomes more acidic (see Figure 1).

The methodology for the use of pHrodo™ BioParticles™ conjugates was developed using whole blood cells. The methodology can also be used with adherent RAW and MMM (J774A.1) murine macrophage cells (Wan, 1993), or adapted for use with other adherent cells, primary cells, or even cells in suspension (Bassøe, 2002). Cells assayed for phagocytic activity with pHrodo™ BioParticles™ conjugates can be fixed with standard 2–4% paraformaldehyde solutions for later analysis, preserving differences in signal between control and experimental samples with high fidelity for up to 48 hours. pHrodo™ BioParticles™ conjugate preparations are also amenable to opsonization, which can greatly enhance their uptake and signal strength in the assay.

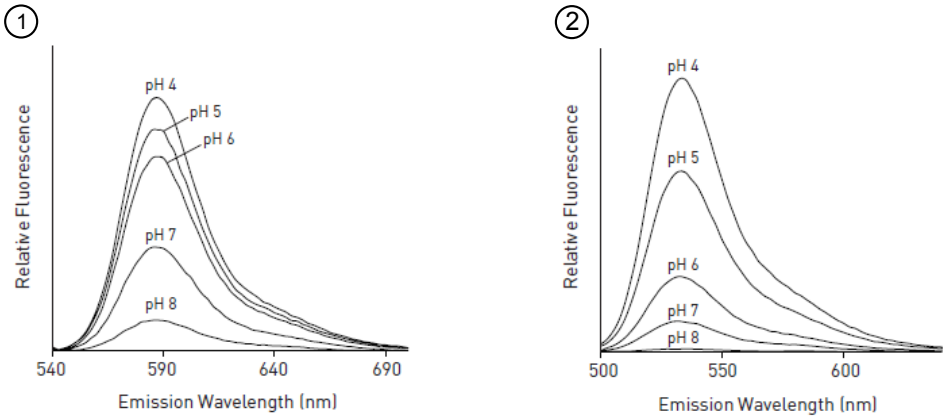

Figure 1 Fluorescence emission spectra of the pHrodo™ BioParticles™ conjugates

① pHrodo™ Red BioParticles™ conjugate ② pHrodo™ Green BioParticles™ conjugates

Procedure overview

The pHrodo™ BioParticles™ phagocytosis assays investigate particle attachment and internalization in order to measure phagocyte activity.

The phagocytic function of polymorphonuclear leukocytes (PMNs) and macrophages is evaluated in the whole blood sample by exposing the heparinized whole blood sample to pHrodo™ BioParticles™ conjugates for at least 15 minutes at 37°C. A control sample containing all reagents is incubated on ice. The phagocytosis is stopped by transferring the samples to ice. The red blood cells or erythrocytes are lysed using the proprietary Lysis Buffer A (Component A) and Buffer B (Component B), followed by centrifugation and washing. The final cell pellet containing white blood cells is resuspended in Wash Buffer (Component C) and is ready for analysis using a flow cytometer equipped with a 488-nm argon-ion laser.

The nucleated phagocytes are distinguished from debris by gating on the granulocyte and monocyte populations using forward and scatter properties. The percentage of active phagocytes is determined by further gating on orange fluorescence signals collected with an R-phycoerythrin emission filter (such as a 585/26-nm band pass filter) for the pHrodo™ Red *E. coli* BioParticles™ Conjugate, or a FITC emission filter (such as a 530/30-nm band pass filter) for the pHrodo™ Green *E. coli* BioParticles™ Conjugate and the pHrodo™ Green *S. aureus* BioParticles™ Conjugate. pHrodo™ BioParticles™ conjugates are novel, no-wash fluorogenic reagents that are non- or weakly-fluorogenic when attached to the outer surface of the phagocyte, but highly fluorescent in the acidic environment of the phagosome upon internalization. This property of pHrodo™ dyes eliminates the washing and quenching steps that are associated with other phagocytosis assay protocols.

Contents and storage

Table 1 pHrodo™ BioParticles™ Phagocytosis Kits for Flow Cytometry

Contents	Amount[1]	Storage [2]
Lysis Buffer A (Component A)	10 mL	• 2°C to 8°C • Do not freeze.
Buffer B (Component B)	200 mL	
Wash Buffer (Component C)	200 mL	
pHrodo™ BioParticles™ conjugate, one of the following depending upon the product that was purchased:		
pHrodo™ Red *E. coli* BioParticles™ Conjugate (Component D) (Cat. No. A10025)	1 vial of lyophilized product	• 2°C to 8°C • Desiccate. • Protect from light.
pHrodo™ Green *E. coli* BioParticles™ Conjugate (Component D) (Cat. No. P35381)	5 vials of lyophilized product	
pHrodo™ Green *S. aureus* BioParticles™ Conjugate (Component D) (Cat. No. P35382)	5 vials of lyophilized product	

[1] Sufficient for 100 assays when using 100-µL whole blood sample volume per assay.
[2] The product is stable for at least 6 months when stored as directed.

Approximate fluorescence excitation and emission maxima of pHrodo™ BioParticles™ conjugates:

Conjugate	Excitation and emission maxima
pHrodo™ Red *E. coli* BioParticles™ Conjugate	560/585 nm
pHrodo™ Green *E. coli* BioParticles™ Conjugate	509/533 nm
pHrodo™ Green *S. aureus* BioParticles™ Conjugate	509/533 nm

Note: pHrodo™ BioParticles™ conjugates are compatible with 488-nm argon-ion laser excitation.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Instrument	
Flow cytometer with 488-nm excitation wavelength (argon-ion laser)	Contact your local sales office.
Equipment	
Water bath or incubator set to 37°C	MLS
Ice bath/bucket	MLS
Water bath sonicator	MLS
Centrifuge	MLS
Consumables	
Sodium heparin blood collection tubes	MLS
Analysis tubes appropriate for the flow cytometer used	MLS
Biohazard wipes	MLS

Procedural guidelines

- Consider blood samples as potentially infectious. Wear a laboratory coat, disposable gloves, and eye protection when handling samples.
- Dispose of blood samples and containers in accordance with local biohazardous waste requirements.
- Heparin is the only anticoagulant that has been tested with the pHrodo™ BioParticles™ conjugates. Do not use other anticoagulants for blood collection.

Before you begin

Prepare Lysis Buffer A and Buffer B

Equilibrate Lysis Buffer A (Component A) and Buffer B (Component B) to room temperature before use.

Reconstitute the pHrodo™ BioParticles™ conjugates

1. Reconstitute the pHrodo™ BioParticles™ conjugates as described.

For...	Do this...
pHrodo™ Red *E. coli* BioParticles™ Conjugate (Cat. No. A10025)	Reconstitute the vial of lyophilized product in 2.2 mL of Buffer B (Component B).
pHrodo™ Green *E. coli* BioParticles™ Conjugate (Cat. No. P35381) or pHrodo™ Green *S. aureus* BioParticles™ Conjugate (Cat. No. P35382)	Reconstitute each vial of lyophilized product in 440 µL of Buffer B (Component B).

   This provides sufficient pHrodo™ BioParticles™ conjugate in a 20 µL aliquot for a 20:1 particle-to-phagocyte ratio (Bassøe, 2002).

2. Vortex for 1 minute, then sonicate for 5 minutes or until all of the fluorescent particles are evenly dispersed.
3. Place the reconstituted pHrodo™ BioParticles™ conjugate on ice for approximately 10 minutes before use.

Collect whole blood samples

1. Collect whole blood samples in blood collection tubes that contain heparin anticoagulant.

   Note: Whole blood samples can be collected, then stored on ice or at 4°C for up to 24 hours before use in the phagocytosis assay.

2. Place the blood sample tubes on ice to cool the samples for 10 minutes before use.

Perform the phagocytosis assay

- Each set of control tubes and experimental tubes are incubated at 4°C (ice) or 37°C.
- We recommend performing the assay with replicates.

1. Prepare four control tubes for each set of experimental samples. Aliquot whole blood and pHrodo™ BioParticles™ conjugate to flow cytometry tubes as described, then vortex briefly.

Tube	Name	Whole blood sample	pHrodo™ BioParticles™ conjugate
1	Negative control on ice	100 µL	—
2	Negative control at 37°C	100 µL	—
3	Positive control on ice	100 µL	20 µL
4	Positive control at 37°C	100 µL	20 µL

2. For each experimental sample, prepare two tubes containing 100 µL of whole blood and 20 µL of pHrodo™ BioParticles™ conjugate, then vortex briefly.
3. Place tubes 1 and 3 on ice, and tubes 2 and 4 in a 37°C water bath, then incubate for 15 minutes.

   For each experimental sample, place one tube on ice and the other tube in a 37°C water bath, then incubate for 15 minutes.

4. Remove all tubes from the 37°C water bath, then place the tubes on ice to stop phagocytosis.
5. Add 100 µL of Lysis Buffer A (Component A) to each tube, vortex briefly, then incubate for 5 minutes at room temperature.

6. Add 1 mL of Buffer B (Component B) to each tube, vortex briefly, then incubate for 5 minutes at room temperature.
7. Centrifuge the samples at 350 × *g* at room temperature for 5 minutes.
8. Discard the supernatant. If necessary, wick the tops of the tubes with biohazard wipes to absorb any residual liquid.
9. Resuspend each cell pellet with 1 mL of Wash Buffer (Component C).
10. *(Optional)* Proceed to one of the following optional staining procedures.
    - For DNA staining—see "(Optional) Perform DNA staining" on page 4.
    - For antibody labeling—see "(Optional) Perform antibody labeling" on page 4.
11. Repeat step 7 and step 8.
12. Resuspend each cell pellet with 0.5 mL of Wash Buffer (Component C).
13. Proceed to analyze the results using flow cytometry (see "Set up the flow cytometer, then analyze the results" on page 5).

(Optional) Perform DNA staining

Perform DNA staining just prior to flow cytometry analysis. DNA staining can improve the separation of debris from events containing DNA.

1. Prepare the DNA staining solution (enough to add 0.5 mL to each sample) by diluting the DNA stain of choice (such as SYTO™ 9 Green Fluorescent Nucleic Acid Stain, Cat. No. S34854) to 100 nM in Wash Buffer (Component C).
2. Centrifuge the samples at 350 × *g* at room temperature for 5 minutes.
3. Discard the supernatant. If necessary, wick the tops of the tubes with biohazard wipes to absorb any residual liquid.
4. Resuspend each cell pellet with 0.5 mL of DNA staining solution.
5. Incubate the samples at room temperature for 15 minutes protected from light.
6. Proceed to analyze the results using flow cytometry (see "Set up the flow cytometer, then analyze the results" on page 5).

   Ensure the emission spectra are appropriately separated, and the recommended emission filters are used.

(Optional) Perform antibody labeling

Perform antibody labeling just prior to flow cytometry analysis to identify subpopulations of white blood cells, or label other relevant surface markers.

1. Centrifuge the samples at 350 × *g* at room temperature for 5 minutes.
2. Discard the supernatant. If necessary, wick the tops of the tubes with biohazard wipes to absorb any residual liquid.
3. Resuspend the samples in antibody labeling buffer that is appropriate for the antibody (not provided).
4. Add the antibody to the samples at the appropriate dilution.
5. Incubate the samples according to the conditions recommended by the antibody manufacturer.
6. Wash the samples with labeling buffer, then centrifuge at 350 × *g*.
7. Resuspend the samples in 0.5 mL of Wash Buffer (Component C) or DNA staining solution (see "(Optional) Perform DNA staining" on page 4, step 1). Perform DNA staining as described (see "(Optional) Perform DNA staining" on page 4).
8. Proceed to analyze the results using flow cytometry (see "Set up the flow cytometer, then analyze the results" on page 5).

   Ensure the emission spectra are appropriately separated, and the recommended emission filters are used.

(Optional) Opsonization

Our studies indicate that pHrodo™ *E. coli* and *S. aureus* BioParticles™ conjugates do not require pre-opsonization for optimal uptake when used with normal whole blood samples. The serum in the whole blood sample is sufficient for opsonization. However, if you are using isolated white blood cells (WBC) or your sample requires the use of an opsonizing reagent, we recommend using the *Escherichia coli* BioParticles™ Opsonizing Reagent (Cat. No. E2870) or *Staphylococcus aureus* BioParticles™ Opsonizing Reagent (Cat. No.S2860). For instructions on using the BioParticles™ opsonizing reagents, refer to the instructions supplied with the reagent.

Set up the flow cytometer, then analyze the results

1. Perform the analysis using a flow cytometer that is equipped with a 488-nm argon-ion laser using a 585/26-nm emission filter for the pHrodo™ Red BioParticles™ conjugates or 530/30-nm emission filter for the pHrodo™ Green BioParticles™ conjugates.

   If additional staining is performed on the samples as described, ensure the emission spectra are appropriately separated, and the recommended emission filters are used.

2. To analyze samples, set up two dot plots: one showing forward scatter (FSC) vs. side scatter (SSC) (see Figure 2), and another showing FSC vs. fluorescence (see Figure 3).

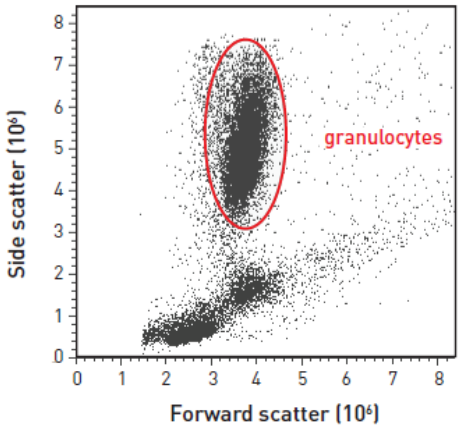

Figure 2 Whole blood sample: FSC vs. SSC

A whole blood sample was processed according to the basic protocol and applied to an Attune™ Acoustic Focusing Cytometer equipped with a 488-nm argon-ion laser. The forward scatter (FSC) and side scatter (SSC) plot shows the selected granulocyte population. This region was used for gating in Figure 3-2 and Figure 4.

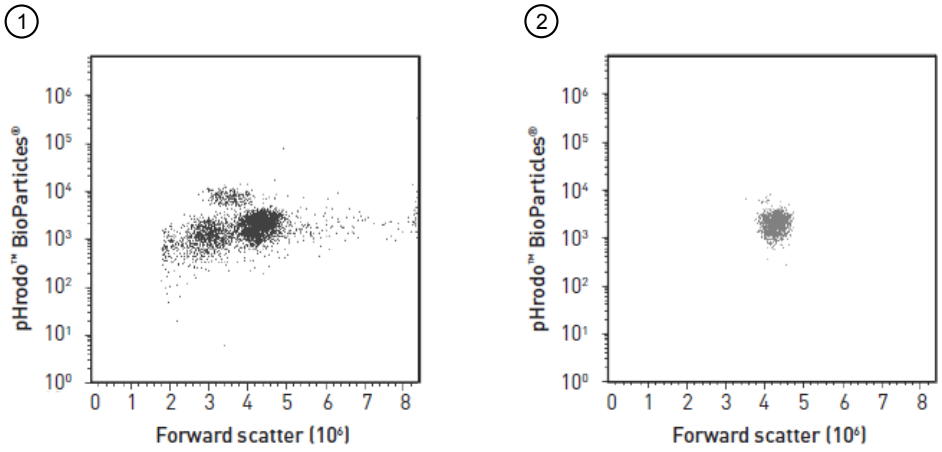

Figure 3 Whole blood sample: FSC vs. fluorescence

The same sample as shown in Figure 2 with the pHrodo™ fluorescence signal on the Y-axis.

① Fluorescent intensities of the various white blood cell populations ② Fluorescence signal of the gated granulocyte population alone

3. Apply a negative control sample (whole blood aliquot without pHrodo™ BioParticles™ conjugates that went through the lysis procedure, tubes 1 or 2), and set linear FSC and SSC voltages to locate the white blood cell scatter pattern as in Figure 2. For FSC vs. fluorescence, set fluorescence on a log scale and set events in the lowest decade. Adjust threshold(s) to eliminate debris.

4. Draw a region around the granulocyte population as shown in Figure 2, and apply the gate as in Figure 3. Adjust the fluorescence PMT (photomultiplier tube) voltage if necessary.

5. Apply the positive control sample (tube 3) or an experimental sample incubated at 37°C to locate the granulocyte population as in Figure 2, apply to Figure 3, then observe the difference in fluorescence between the negative control sample and the positive control or experimental sample. Adjust the fluorescence PMT voltage if necessary.

6. Apply the positive control sample (tube 3, which includes all reagents, but is incubated on ice) and ensure the events for FSC vs. SSC are on scale.

7. Once the settings are adjusted, apply experimental samples (incubated on ice and at 37°C), then collect the appropriate number of events.

8. Perform gating on FSC vs. SSC, and FSC vs. fluorescence as described above.

9. Generate and record statistics for experimental samples incubated on ice and at 37°C.

   You should observe good white blood scatter pattern on FSC vs. SSC, greater than 96% phagocytosing neutrophils (for normal whole blood samples), and greater than 5-fold increase in fluorescence signals between the control and experimental samples. The desired statistics may be % phagocytosing neutrophils, granulocytes, or monocytes, or the fluorescence signal of various samples. For an example of expected results, see Figure 4.

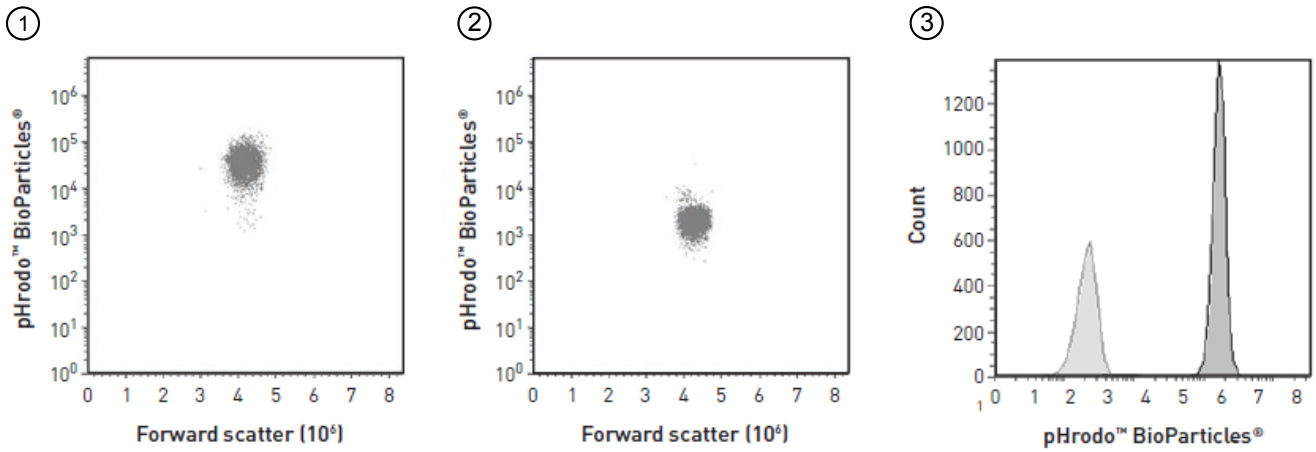

Figure 4 Fluorescent intensities of pHrodo™-stained white blood cell populations

① The same figure as Figure 3, showing a population of granulocytes that have phagocytosed pHrodo™ BioParticles™ conjugates.

② A control sample in which phagocytosis was inhibited by incubating on ice, demonstrating the lack of fluorescence because pHrodo™ BioParticles™ conjugates were not in acidic phagosome compartments, and any attached particles show very low fluorescence.

③ A histogram overlay of (1) and (2) showing the fluorescence separation between the experimental and negative control sample.

Troubleshooting

Observation	Possible cause	Recommended action
Poor white blood cell scatter	Lysis was incomplete.	Perform lysis for 5 minutes at room temperature. Lysis is complete when the solution is translucent.
		Ensure Lysis Buffer A (Component A) and Buffer B (Component B) are equilibrated to room temperature before use.
	The instrument was not set up correctly.	Check the voltage and particle size setting on the flow cytometer.
Low fluorescence of particles or low percent phagocytosing cells	The assay conditions were incorrect.	It can be a normal result due to the condition of the serum or cells. If the uptake is expected to be high, then do the following: • Perform the phagocytosis assay at 37°C for at least 15 minutes. For opsonized particles, the incubation time may need to be optimized. • Resuspend the lyophilized product in 2.2 mL Buffer B (Component B) to obtain sufficient particle concentration in a 20 µL aliquot for a minimum 20:1 particle-to-phagocytosing cell ratio.
	The phagosome pH was below the pKa of the pHrodo™ dye.	The pKa of the pHrodo™ dye is ~7.3. If the pH of the intracellular compartment is higher than 7.3, there may not be sufficient fluorescence signal to detect.

Related products

Cat. No.	Product name	Amount
A10010	pHrodo™ Red *S. aureus* BioParticles™ Conjugate for Phagocytosis	5 × 2 mg
A10026	pHrodo™ Red Phagocytosis Particle Labeling Kit for Flow Cytometry (100 tests)	1 kit
B7277	Bacteria Counting Kit, for flow cytometry	1 kit
C36950	CountBright™ Absolute Counting Beads (100 tests)	5 mL
E2870	*Escherichia coli* BioParticles™ Opsonizing Reagent	1 unit
F2902	Fc OxyBURST™ Green Assay Reagent	500 µL
L34856	LIVE/DEAD™ *Bac*Light™ Bacterial Viability and Counting Kit, for flow cytometry	1 kit
O13291	OxyBURST™ Green H_2HFF BSA - Special Packaging	5 × 1 mg
P35361	pHrodo™ Red *E. coli* BioParticles™ Conjugate for Phagocytosis	5 × 2 mg
P35364	pHrodo™ Red Zymosan BioParticles™ Conjugate for Phagocytosis	5 × 1 mg
P35365	pHrodo™ Green Zymosan BioParticles™ Conjugate for Phagocytosis	5 × 1 mg
P35366	pHrodo™ Green *E. coli* BioParticles™ Conjugate for Phagocytosis	5 × 2 mg
P35367	pHrodo™ Green *S. aureus* BioParticles™ Conjugate for Phagocytosis	5 × 2 mg
P36600	pHrodo™ Red, succinimidyl ester (pHrodo™ Red, SE)	1 mg
S2860	*Staphylococcus aureus* BioParticles™ Opsonizing Reagent	1 unit
S7572	SYTO™ Green Fluorescent Nucleic Acid Stain Sampler Kit #1 (50 µL each of SYTO™ dyes 11-16)	1 kit
S34854	SYTO™ 9 Green Fluorescent Nucleic Acid Stain (5 mM solution in DMSO)	100 µL

For a complete list of antibodies for flow cytometry, go to **thermofisher.com/flowcytometry**.

Limited product warranty

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References

Sahlin S, Hed J, Rundquist I (1983) Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay. *J Immunol Methods* 60(1–2):115–124.

Wan CP, Park CS, Lau BH (1993) A rapid and simple microfluorometric phagocytosis assay. *J Immunol Methods* 162(1):1–7.

Bassøe C (2002) Assessment of Phagocyte Functions by Flow Cytometry. *Current Protocols in Cytometry* 21(1):9.19.1–9.19.22.

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

Revision history: Pub. No. MAN0001950

Revision	Date	Description
B.0	11 December 2019	• Corrected the procedure for reconstituting the lyophilized pHrodo™ BioParticles™ conjugates with Buffer B (Component B). • Made minor edits for style and clarification.
A.0	30 October 2013	Baseline for this revision history.